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URINARY TUMOR ASSOCIATED ANTIGEN,
ANTIGENIC SUBUNITS AND METHODS OF DETECTION

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URINARY TUMOR ASSOCIATED ANTIGEN,
ANTIGENIC SUBUNITS USES AND METHODS OF DETECTION

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BACKGROUND OF THE INVENTION

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This invention relates generally to tumor-associated
antigens, specifically to an antigen found in the urine of
cancer patients which can be used for immunodiagnosis,
immunoprognois, and therapy of human cancer.

15

It is well documented in animal models that cells were
changed biochemically and morphologically after neoplastic
transformation in vivo. Such transformed neoplastic cells
in appropriate doses are capable of inducing protective
20 immunity against tumor development in syngeneic animals
when subsequently inoculated with adequate number of viable
neoplastic (cancer) cells. The protective immunity was
determined to be due to emergence of certain new components
that were called tumor-specific transplantation antigens.
25 Expression of similar components, so called tumor-
associated antigens, by human malignant tumor cells has
been identified by serologic analysis using autologous and
allogeneic human sera as the source of antibody. Use of
sera from animals immunized with human cancer cells and
30 murine monoclonal antibodies developed against human tumors
have added to the definition of additional tumor-associated
antigens. However, xeno-polyclonal or murine monoclonal
antibody defined antigens on human tumor cells are not
necessarily immunogenic in humans. While the physico-
35 chemical properties of almost all of the antigens defined
by xeno-polyclonal and murine monoclonal antibodies have
been elucidated in great detail, such information is
available for only a very few tumor-associated antigens

defined by autologous and allogeneic antibodies. The reasons for such paucity have been difficulties in solubilizing adequate amount of the antigens for subsequent purification to homogeneity and the polyclonal nature of autologous and allogeneic antibodies. Without the availability of well characterized tumor-associated antigens that are immunogenic in the host, the importance of these antigens is not fully realized in terms of their application for immunodiagnosis, immunoprognosis and treatment of human cancer.

The tumor-associated antigens in human neoplasms that have been defined by autologous and allogeneic antibodies vary in their distributions. Some are expressed only by individual tumor cell lines or tumors; some are shared by histologically dissimilar tumors including organs where the tumor arises and fetal tissues. The antigens that are expressed only by individual tumors are of limited importance for immunodiagnosis and treatment because tumor cell lines generally do not establish from every single tumor and cannot be applied to another patient. On the contrary, tumor antigens that are shared by different tumors of the same histologic type or by histologically dissimilar tumors can be applied for immunodiagnosis, immunoprognosis and treatment of different patients with different types of cancers.

There are well documented instances to suggest that immunity against growing neoplasm in humans can be enhanced by active immunization with antigen bearing tumor cells. The purpose of such active specific immunotherapy is directed at attempts to enhance the level of anti-tumor immunity beyond that which is naturally induced by the growing neoplasm. It is believed that a growing neoplasm does not induce a maximum immune response in the host to the tumor associated antigens it contains. Most immunotherapy attempts have involved vaccines prepared from

whole tumor cells, because progress has been slow in the isolation and purification of human tumor associated antigens. The possibility that living autologous tumor cells could result in tumor growth at the inoculation site has inhibited the use of such vaccines in man. However, tumor cells that express high levels of shared common tumor-associated antigens can be used to immunize different patients (Morton, D.L. et al, In Terry, W.D., Rosenberg, S.A. (eds): Immunotherapy of Human Cancer. New York, Elsevier North Holland, pp 245-249 (1982); Livingston P.O., et al., Int. J. Cancer 31:567 (1983)). The advantage of using such an allogeneic vaccine is two-fold: (1) an immune response induced against the foreign HLA transplantation antigens on the allogeneic vaccinated tumor cells would cause their rejection; (2) this immunization should induce a strong immune response against the shared common cross-reacting tumor-associated antigens to which the human leukocyte antigens (HLA) might serve as a helper function.

Most attempts at immunotherapy in man have been with vaccines composed of inactivated tumor cells, crude extracts or preparations from isolated membranes. Although such preparations may be effective in eliminating progressive tumor growth, there is always the great danger of inactivating the tumor-associated antigens during preparation of the vaccine, unless immunologic reagents and sensitive techniques are available for systemically following the isolation-purification procedure.

Cancer patients who are most likely to respond to an active specific immunotherapy are those who are early in their disease and have minimal residual tumor burden following treatment with other therapeutic modalities (Morton D.L., Seminars in Oncology 13:180 (1986)).

Using lyophilized and reconstituted urine samples of cancer patients and autologous serum as the source of

antibody in a complement fixation assay, immunologic reactivity has been observed. The reactivity was abolished by absorption of the sera with tumor cells and not by human normal ~~cell~~^{cells}. These observations indicated that immunologically similar antigens were present in the urine samples and tumor cells. Furthermore, the observed reactivity in urine samples of cancer patients who were studied sequentially disappeared after surgical ablation of tumor but reappeared before tumor recurrence, (Gupta, R.K. et al., J. Surg. Oncol., 11:65 (1979)). Because many of the test samples were highly anti-complementary, perhaps due to artifacts arising during lyophilization process, a different method was developed for preparing urine samples for testing. In this study, 24 hour urine samples were obtained from larger numbers of cancer patients and normal controls. The urine samples were concentrated 100-fold by centrifugation and ultrafiltration, and tested by complement fixation using autologous serum as the source of antibody. Ninety-two percent (55/60) of cancer patients were positive for the antigens in their urine as opposed to only 7% (2/27) normal controls. Antibody activity of the sera reacting to the urine from cancer patients was removed by absorption with biopsied tumor specimens but not with normal skin or muscle suggesting that the antigens detected in urine of cancer patients were tumor associated (Rote, N.S. et al, J. Surg. Res. 29:18 (1980)).

^a
In subsequent investigation, an allogeneic serum that had high titer to antigens in urine was used as the source of antibody. Use of this antibody source in complement fixation revealed that urine samples of 94.7% of cancer patients and 35.1% of normal controls were positive. Again, absorption of the allogeneic serum with tumor cells (autologous to the urine source) removed the antibody activity. However, human normal lymphocytes, skin and muscle cells were ineffective as absorbents. Furthermore, excretion of antigens into urine appeared to depend on the

presence of tumor in the patients, because removal of tumor by curative surgery resulted in cessation of the putative antigens excretion. The urine remained negative as long as the patient was free of tumor (Rote, N.S. et al., Int. J. Cancer 26:203 (1980)). However, presence of the antigens in 35% of normal urine indicated a cross-reacting antigenic system which prevented this test from practical use.

Gel filtration chromatography of the concentrated urine revealed that the antigenic activity was present in the first peak of the elution profile. However, when this procedure was performed in the presence of 6M urea, the antigenic activity was found in three different peaks representing various molecular sizes, ^{the} majority of the activity being in the first peak. However, because of the polyclonal nature of the allogeneic serum that was used as the source of antibody, it was impossible to determine if the antigenic activity in different peaks represented disassociation product of a large antigenic complex bearing the same epitope or represented different epitopes. Similar results were observed when lyophilized and reconstituted urine was used (Rote, N.S. et al, supra). Thus the antigens in urine of cancer patients recognized by autologous and allogeneic antibody was actually a macromolecular complex and because of polyclonal nature of the antibody, the nature of the specific epitope could not be determined. However, the majority of the evidence suggested that the excretion of antigenic macromolecular complex into urine of cancer patients was dependent on the presence of tumor in the cancer host. Serial measurements of tumor-associated antigens in the urine of cancer patients who received preoperative chemo- and radiation therapy were made by complement fixation. The level of excretion of the antigens into urine as a result of therapy were compared to pretreatment samples and changes were correlated with clinicopathological evidence of in situ tumor cell destruction. Of the 53 cancer patient studied

in this manner, 44 had clinicopathologic evidence of tumor destruction induced by the preoperative therapy, and all 44 patients had four-fold or greater rise in the level of urinary antigens during the treatment period. The other
5 nine patients had no evidence of tumor destruction and the antigen titers in these patients remained unchanged. These results suggested that excretion of tumor-associated antigens in urine could be used to ~~asses~~^{assess} the in vivo effectiveness of tumoricidal therapy of nonaccessible
10 tumors (Huth, J.F. et al., Cancer Treat. Rep. 65:1037 (1981)). Similar results were observed in patients with colon carcinoma receiving hyperthermia and chemotherapy. Again, the incidence of antigenic activity in urine of apparently healthy individuals was high, i.e. 10% (2/20).
15 (Fink, S.J. et al., J. Surg. Oncol. 21:81 (1982)).

Because allogeneic serum was used as the source of antibody in the complement fixation assay, the possibility existed that part of the immunologic reactivity with urine
20 samples could be due to histocompatibility antigens. Therefore, the serum was absorbed with pooled lymphocytes to remove as much anti-HLA antibodies as possible from the serum. This often added anticomplementary activity to the serum. This problem was obviated by using the serum at a
25 dilution beyond the anticomplementary activity level. However, this resulted in reduced sensitivity of the assay. Furthermore, some of the test (urine) samples by themselves exhibited the anticomplementary activity rendering them unsuitable for detection of urinary antigens by complement
30 fixation. To circumvent these problems, a competitive inhibition enzyme immunoassay was developed. In this assay, reactivity between known amounts of autologous antibody and tumor-associated urinary antigens was competitively inhibited by allogeneic urine (test) samples
35 only if the test samples contained immunologically similar antigens. The results of the assay correlated very well with the results of complement fixation without having to

deal with the problem of anticomplementary activity and reactivity due to HLA present in the test urine samples. However, the test lacked specificity because of reactivity with urine of normal individuals. (Huth, J.F. et al.,
5 Cancer 47:2856 (1981)).

Analysis of urinary tumor-associated antigens by gel-filtration chromatography consistently revealed that the antigenic complex recognized by autologous and allogeneic
10 antibodies had a molecular mass of greater than 300 kD. This antigenic mass was clearly too large to pass through the glomerular basement membrane of kidney by simple diffusion. There are several reports in literature concerning the development of nephrotic syndrome in cancer
15 patients. Renal biopsies of these patients often demonstrated the deposition of immune complexes within the glomerular basement membrane (Laughridge, L.W. and Lewis, M.G., Lancet 1:256 (1971); Couser, W.G. et al., Am. J. Med. 57:962 (1974)). Thus, it was logical to assume that
20 antigens shed by tumor cells in vivo into circulation would react with specific antibodies to form circulating immune complexes. These immune complexes might deposit in the glomerular basement membrane and cause membrane damage that would allow the passage of high molecular weight antigenic
25 complex into the urine. A relationship between antigen nonspecific immune complexes in circulation of cancer patients and excretion of urinary antigens was observed. Of 36 cancer patients who were positive for urinary antigens, 28 (78%) were also positive for circulating
30 immune complexes at the time of urine collection. Of 24 patients that were negative for circulating immune complexes, 22 (92%) were also negative for urinary antigens. In a cancer patient whose serum and urine samples were studied sequentially during this course of
35 thermochemotherapy, fluctuations in the levels of circulating immune complexes and excretion of urinary antigens were parallel. These results suggested that

excretion of urinary tumor-associated antigens into urine of cancer patients was not an isolated phenomenon; rather, immune complex deposition in kidneys appeared to cause glomerular damage which allowed passage of the antigens into the urine, Huth, J.F. et al., Cancer 49:1150, (1982).

In an attempt to determine the applicability of the urinary tumor-associated antigens for prognostication of cancer patients, the antigenic complex was partially purified and used as target antigen in the competitive inhibition enzyme immunoassay. One hundred-fold concentrated urine samples from normal controls and melanoma patients were used to establish the base line, distribution of the antigens, and early detection of subclinical recurrence. The results were expressed as antigen units (ng antigenic protein/mg creatinine/24 hours) for comparison among individuals. The antigen levels in urine of melanoma patients (median = 56.5 units, n = 56) were significantly higher ($p < 0.05$) than those of normal controls (median = 1.9 units, n = 56). The 90th percentile for the normal group was 34.3 antigen units. Using this value as the criterion for positivity, 64% (36/56) urine samples of melanoma group were positive for the antigens as opposed to 11% (6/56) of normal controls. Subsequently a retrospective analysis of 58 melanoma patients paired on the basis of disease recurrence and no recurrence after lymphadenectomy revealed a median antigen level of 68 units for the recurrent group and 18.9 for the non-recurrent group. Eighteen of 29 (62%) melanoma patients who had recurrence of their disease and 9 of 29 (31%) patients who remained disease free were urinary antigen positive. These incidences were significantly different ($p < 0.005$) (Gupta, R.K. et al., Diagnostic Immunol. 1:303 (1983)). Though the results of the above investigations confirmed previous observations, the utility of urinary antigen detection assay remained uncertain because of the consistent observation that urine of many (11%) normal individuals had

considerable levels of the antigen.

Despite significant progress made in developing assays using tumor markers that are not immunogenic in the cancer host, e.g., CEA, alpha-fetoprotein, prostate specific antigen, etc., there exists a need to diagnose and treat tumors using tumor-associated antigens that are immunogenic in the cancer host. This invention satisfies these needs by providing for the detection of various tumors by detecting U-TAA while avoiding the detection of false positives. In addition, this invention provides antigenic subunits of U-TAA and a vaccine which induces cell mediated specificity for individual determinants on the tumor cell surface, as well as anti-U-TAA antibody production.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows Sephacryl S-200 gel filtration chromatogram of concentrated melanoma urine. Protein content (absorbance 280 nm) and U-TAA activity (ng/100 μ l) determined by an allogeneic double determinant EIA are shown.

Figure 2 shows the purification of baboon IgG and IgM antibodies to U-TAA by DEAE Affi-Gel Blue chromatography. The IgG anti-U-TAA antibody was eluted first and IgM anti-U-TAA antibody was eluted with 0.5M NaCl at a flow rate of 60 ml/hr. The pool of fractions comprising IgG had a protein concentration of 3.5 mg/ml and anti-U-TAA titers of 1:250,000. The pool of IgM fractions had a protein concentration of 3.7 mg/ml and anti-U-TAA titer of 1:3600 using purified U-TAA (30 ng/well) as the target.

Figure 3 shows the effect of absorption of baboon anti-U-TAA IgM antibody by UCLA-SO-M14 (M14) and autologous lymphoblastoid (L14) cells on cytolysis in a complement dependent cytotoxicity assay. The cytotoxic effect of

baboon IgM anti-U-TAA was completely abolished by absorption with 1×10^7 M14 cells and was not affected significantly by the same number of L14 cells.

5 Figure 4 shows the effect on baboon IgM anti-U-TAA antibody complement dependent assay (CDC) by purified U-TAA. The antibody (3.7 mg/ml) was diluted by RPMI-10% FCS and mixed with an equal volume of U-TAA (0.06 mg/ml) so as to arrive at the indicated final dilution. Purified U-TAA
10 significantly reduced the cytotoxicity at each dilution of the antibody.

Figure 5 shows DEAE Sephacel anion exchange chromatogram of AD1-40F4 reactive melanoma serum. Protein
15 content (absorbance 280 nm), presence of IgG, presence of IgM, and U-TAA activity (O.D._{405nm}) as determined by a xenogeneic double determinant EIA are shown.

Figure 6 shows U-TAA purified from the serum of a
20 melanoma patient was separated by SDS-PAGE and then transferred to nitrocellulose paper. Both murine monoclonal and polyclonal baboon anti-U-TAA antibodies recognize a 90 kDa subunit.

25 Figure 7 shows SDS-PAGE profile of immunoprecipitates formed by reacting NP-40 extract of intrinsically labeled (¹⁴C-L-leucine) melanoma (UCLA-SO-M14) cells with human polyclonal antibody. The antibody was used before (A) and after (B) pretreatment with purified U-TAA.

30

Figure 8 shows isoelectric focusing of U-TAA purified from the serum of a melanoma patient. After isoelectric focusing, the proteins were blotted onto nitrocellulose and stained with baboon polyclonal anti-U-TAA antisera. A
35 single band corresponding to an isoelectric point of 6.1 was identified.

Figure 9 shows the time difference between the appearance of U-TAA in the urine of surgically treated melanoma patients and the clinically detectable recurrence of the disease.

5

Figure 10 shows the effect of gamma and alpha interferon on the level of U-TAA expression by three melanoma cell lines (UCLA-SO-M10, UCLA-SO-M24, and UCLA-SO-M101). Cells were cultured in RPMI-10% FCS medium with
10 and without 500 units/ml of gamma and alpha interferon for 96 hours. U-TAA level was determined by the competitive ELISA using allogeneic antibody and purified U-TAA (0.06 mg/ml).

15 Figure 11 shows inhibition of reactivity as observed by indirect membrane immunofluorescence between allogeneic anti-U-TAA serum and UCLA-SO-M24 (melanoma) cells by purified U-TAA. The anti-U-TAA serum at varying dilutions was preincubated with 1.0 μ g U-TAA and then reacted with
20 the melanoma cells. While the untreated anti-serum maintained reactivity at 1:16 dilution (\square), it was blocked by 93% after preincubation of the serum with purified U-TAA (\blacksquare).

25 Figure 12 shows the continued production of anti-U-TAA IgM antibody by one of the human lymphoblastoid cell lines (LCL 2) for more than six months. Cells were grown in RPMI-FCS medium. Culture supernates were collected at regular time intervals and tested in the direct ELISA using
30 purified U-TAA (30 ng/well) for the presence of both IgG and IgM antibodies. The cultures produced IgM antibody only.

35 Figure 13 shows Sephacryl S-200 elution profile of cell free NP-40 extract of intrinsically labeled (14 C-L-leucine) UCLA-SO-M14 (melanoma) cells and reactivity of various peaks with culture supernate of LCL 2 human

lymphoblastoid cells. One hundred microliters of each pool was reacted with 100 μ l of LCL 2 culture supernate at 37°C for one hour. Antibody bound radioactivity was separated from the unbound radioactivity by rabbit anti human Ig
5 immunobeads. To reduce the background, the cell free NP-40 extract was pretreated with the immunobeads. The antigenic activity was present in the peak encompassing fraction 39 through 45.

10 Figure 14 shows that reactivity of LCL 2.6 culture supernate to U-TAA was blocked by U-TAA and not by FA. The supernate was reacted to U-TAA in the direct ELISA before and after blocking either with 100 μ l (6 μ g) of U-TAA or 100 μ l (5 μ g) of FA at each dilution of the supernate.

15

Figure 15 shows that the reactivity of LCL 2.6 culture supernate to FA was blocked by FA and not by U-TAA. The blocking was performed as described in Figure 14.

20 Figure 16 illustrates schematic conceptualization of the U-TAA specific immune complex detection assay. This utilizes anti-U-TAA murine monoclonal antibody, AD1-40F4. Immune complexes react with the immobilized antibody (anti-U-TAA). The capture of human immunoglobulin via the
25 antigen is determined by the use of rabbit or goat anti-human Ig in a manner similar to ELISA.

Figure 17 shows correlation between U-TAA specific immune complex level (OD_{405nm}) and clinical course
30 (recurrence) in patients with melanoma. The patients with stage II disease were rendered disease free by surgical resection of the primary disease and lymphadenectomy. Serum samples were obtained every time the patient visited the clinic for check-up. The serum samples were analyzed
35 for U-TAA specific immune complexes by the assay outlined in Figure 16. Sera were positive for U-TAA specific immune complexes in patient #1 at least 34 weeks before and in

patient #2 at least 28 weeks before clinically detectable recurrence. NED = no evidence of disease.

DETAILED DESCRIPTION OF THE INVENTION

5

As used herein, "Urinary Tumor-Associated Antigen (U-TAA)" refers to a high molecular weight glycoprotein that was initially detected in the urine of melanoma patients but subsequently found to occur in other body fluids as well.

10

In this invention, the antigen U-TAA has been separated from the majority of normal serum proteins by DEAE Sephacel anion exchange chromatography. Even though U-TAA is immunogenic in man, the majority of antigen eluted free of antibody in the second peak of the column. This observation was not surprising as the sera used in these investigations were selected on the basis of high reactivity with the murine monoclonal antibody. Therefore, it is likely that the patients with such high levels of circulating U-TAA were in relative antigen excess and one would expect to isolate antigen free of immunoglobulins. The small amount of U-TAA seen in association with the major IgG and IgM peaks suggested that some anti-U-TAA antibody was present in the form of immune complexes. All urine samples from melanoma patients that were positive for U-TAA using the allogeneic antibody also reacted with the murine monoclonal antibody. The urine samples that were negative in the allogeneic antibody assay were also negative in the monoclonal antibody assay. Furthermore, the monoclonal reacted with the concentrated and partially purified fraction of UCLA-SO-M-14 cultured melanoma cell supernatant. This suggests that immunologically similar antigens excreted into the urine of melanoma patients are also produced by melanoma cells in culture. The monoclonal reactive molecule in melanoma urines shares many characteristics with the allogeneic antibody-reactive U-

35

TAA.

Mixed glycosidase treatment abolished the U-TAA activity of urine samples from normal individuals as measured by the allo-antibody assay. Similar treatment of specimens from melanoma patients, however, significantly augmented the U-TAA activity in the majority of patients. These observations are consistent with the view that the allo-antibody reactive epitope is a protein which resides on a large, variably glycosylated molecule. In this scenario, glycosidase treatment strips carbohydrate from the molecule, exposing additional immunoreactive epitopes.

This theory is further supported by enzyme digestion of the monoclonal reactive epitope. While treatment with hyaluronidase has little effect on the immunoreactivity of the epitope, protease treatment destroys it. Also mixed glycosidase treatment enhances the immunoreactivity of the molecule.

Numerous murine monoclonal antibodies specific for melanoma have been produced by immunizing mice with whole melanoma cells or cell extracts, for example, (Lloyd, K.O., In Basic and Clinical Tumor Immunology, R. Herberman, ed. Nijhoff, The Hague, pp 159-214 (1983)). More uncommon are monoclonal antibodies specific for allo-immunoreactive proteins (Hadas, E. et al., Cancer Res. 46:5201-5205 (1986))

By screening hybridoma supernatants against a panel of melanoma and normal urines, it was possible to identify one clone with specificity for the tumor associated protein. The resultant antibody recognized a protein epitope which occurs with a much greater frequency in the urine and serum of melanoma patients than in that of normal patients. Therefore, the murine monoclonal antibody is significant for immunodiagnosis and immunoprognosis of human cancer.

The invention provides a substantially purified antigenic polypeptide subunit of urinary tumor-associated antigen (U-TAA) having, after reduction by β -mercaptoethanol and separation by SDS-polyacrylamide gel electrophoresis, a molecular weight of about 90-100 kD (The subunit was about 90 kD from serum and about 100 kD from urine but represents the same subunit since a murine monoclonal antibody recognizes each subunit.) Urinary tumor-associated antigen has been detected in the sera of 64% of disease-bearing melanoma patients, but rarely in the sera from apparently normal individuals. The antigen purified by DEAE Sephacel anion exchange chromatography is heat stable, has a molecular mass in the range of 590-620 kD under non-reducing conditions and an isoelectric point of 6.1. SDS-PAGE under reducing conditions, resolves this high molecular weight antigen into several components.

The epitope recognized by a murine monoclonal antibody AD1-40F4, is present only on 90-100 kD subunit of the urinary tumor-associated antigen. This subunit, however, contains epitopes recognized by baboon and human polyclonal antisera as well. This has been determined by SDS-PAGE immunoblotting. The epitope recognized by the murine monoclonal antibody is different from those recognized by human polyclonal antibodies.

By contacting the U-TAA from a body fluid of an afflicted subject with the murine monoclonal antibody, the amount of U-TAA per a given amount of body fluid can be compared with an amount previously determined for an equivalent sample; a variation in U-TAA indicates a variation in the state of the malignancy. Thus, monitoring a malignancy refers to the process of repeatedly assaying an afflicted subject's body fluids to determine the amount of U-TAA or the 90-100 kD subunit, present in the fluid. Assays may be performed early in treatment of the patient,

as well as during and after treatment. Initially, U-TAA levels may be very high indicating a high turnover or shedding of the antigen. However, after treatment and inhibition of proliferation of tumor cells by vaccination, for example, U-TAA levels in a patient's body fluids may decrease.

The invention allows one to diagnose a tumor in a subject by the method comprising detecting the epitope located on the 45, 65, 90-100, or 120 kD polypeptide from U-TAA in a subject's body fluid after reduction by β -mercaptoethanol and separation by SDS-polyacrylamide gel electrophoresis. The detection can be accomplished by contacting the polypeptide with a reagent and detecting the presence of the reagent which is reactive with the polypeptide.

The invention describes a method of detecting a breast or lung carcinoma in a subject comprising detecting the presence of U-TAA from a sample of the subject. The detection comprises binding the U-TAA with a reagent and detecting the reagent. One example of detection is the binding of U-TAA directly or indirectly by a second reagent. The reagent is preferably an antibody but can be any suitable reagent.

A vaccine is provided for inducing or enhancing antibodies or cell mediated immunity directed against the 90-100 kD polypeptide comprising tumor cells having a U-TAA on the cell surface and at least one tumor associated antigen selected from the group consisting of GM-2, GD-2, Fetal Antigen, or Melanoma-Tumor Associated Antigen, and a pharmaceutically acceptable carrier. Improved results are possible if the tumor cells have HLA of the same type as that of the subject on the cell surface. The vaccine provides a method for inducing or enhancing in a subject afflicted with a cancer the production of antibodies

reactive with the polypeptide subunit of U-TAA having a molecular weight of about 90 to 100 kD, comprising administering to the subject an effective dose of the vaccine. The subject of the present invention is a human
5 being, however, any animal may be used. The antibody produced in the individual after administration of the vaccine inhibits or treats the cancer, for example a melanoma. Inhibiting the cancer refers to the ability to contact the tumor cells with a reagent which can prevent
10 the cells from proliferating, thus resulting in cell death and a reduction in size of the tumor. Alternatively, inhibiting can include a direct cytotoxic effect on the tumor cells.

15 In addition, the invention provides for development of reagents which are reactive with antibodies which are reactive with Urinary Tumor Associated Antigen. These reagents can be anti-idiotypic antibodies which refer to immunoglobulins which bear the internal image of the
20 antigen of interest. Idiotypes are antigenic determinants of the antibody combining site, therefore, anti-idiotypic antibodies mimic the antigenic epitope of an antigen. The invention provides a method of immunotherapy comprising injecting into a subject a therapeutic amount of the anti-
25 idiotypic antibody. The therapeutic amount is any amount effective to produce a cytostatic or cytotoxic effect on the tumor cells which can readily be determined by one skilled in the art.

30 The discovery that U-TAA is found on the surface of tumor cells allows a method of treating a tumor expressing U-TAA on the tumor cell surface in a subject comprising injecting into the subject a tumor inhibiting reagent reactive with U-TAA on the tumor cell surface. The reagent
35 may be an antibody and the antibody may be attached to a cytotoxic or cytostatic agent. The cytotoxic or cytostatic agent, for example may be selected from the group

consisting of a toxin, radiolabeled moiety, and chemotherapeutic agent.

The invention further provides a method of detecting
5 U-TAA on tumor cells from a biopsy comprising contacting
the tumor cells with the murine monoclonal antibody and
detecting the bound antibody. Detection methods for the
presence of nucleic acid or antigen of the present
invention include hybridization of a nucleic acid probe
10 with the nucleic acid of a cell and cell staining with
polyclonal or monoclonal antibodies. Such techniques are
accomplished by methods well-known to those skilled in the
art.

15 A nucleic acid encoding the polypeptide and a nucleic
acid probe capable of selectively hybridizing with the
nucleic acid is also provided. The nucleic acid can encode
an antigenic portion of the epitopes on U-TAA as determined
by the antigenic subunits provided herein. In addition,
20 the nucleic acid can correspond to an antigenic sequence on
an anti-idiotypic antibody and a nucleic acid probe capable
of selectively hybridizing with the nucleic acid
corresponding to the anti-idiotypic.

25 A method of in vivo detection of a tumor in a subject is
provided which comprises injecting into the subject a
reagent, for example an antibody, reactive with U-TAA on
the tumor cell surface, detecting the presence of the
reagent which reacts with the U-TAA and thereby detecting
30 the tumor. The tumor may be for example, a melanoma,
sarcoma, or carcinoma.

The invention still further provides a method for
detecting low levels of U-TAA comprising enhancing the
35 expression of U-TAA in cancer cells with alpha and gamma-
interferon, or other biological response modifiers, e.g.,
retenoic acid, contacting the U-TAA with a reagent and

detecting the presence of the reagent. The use of interferon as an anticancer agent is currently under intensive investigation. Immune or gamma interferon is produced when sensitized lymphocytes are stimulated with
5 specific antigens. Interferon can be administered to a subject by injection as well. Gamma interferon has been shown to induce, enhance or inhibit the expression of several genes. Among those induced are HLA genes including A, B, C. The expression of HLA genes allows certain cells
10 to be more easily recognized and cleared by the immune system. Surprisingly, it has been found that gamma-interferon also enhances expression of U-TAA in some cell lines.

15 The melanoma tumor cell vaccine (MCV) utilizes allogeneic melanoma cell lines which express four well characterized tumor associated antigens, all of which are widely immunogenic in man. The administration of whole irradiated melanoma cells that express U-TAA does induce
20 anti-U-TAA antibodies of both IgG and IgM isotypes. It is notable that 2-to 5-fold increases in anti-U-TAA IgM titers were detected in 11 of the 15 patients, while IgG responses were seen in only 6 of the 15 patients. Why the IgM response is not consistently translated into an IgG
25 response is not readily apparent. It is probable, however, that the polysaccharide moiety of this large glycoprotein molecule induced IgM antibody by T-cell independent mechanisms. This would result in the production of low affinity IgM in small quantities without a subsequent
30 switch to IgG as observed.

With regard to the specificity of the antibodies induced in response to the MCV, the vaccine should elicit antibodies to an array of antigens associated with melanoma
35 cells comprising the vaccine and the adjuvant. U-TAA that was purified from the urine of a melanoma patient and that is expressed on surface of melanoma cells as the target

antigen in ELISA was used. Therefore, antibody activity and its elevation in response to MCV observed in this investigation should be specific to U-TAA. However, to rule out the possibility of non-specific reactivity of the antibodies detected in this investigation, the serum samples from melanoma patients were reacted against KLH as the target antigen. The anti-KLH antibody levels in these patients fluctuated during the course of immunotherapy, but there was no concordance with the elevation of anti-U-TAA antibodies. Furthermore, addition of bovine serum albumin (BSA) to the dilution buffers does affect the antibody levels when U-TAA was used as the target antigen. Thus, despite the polyclonal nature of the serum samples, the elevations in antibody levels observed in this investigation were specific for U-TAA.

A frequent explanation for the transient antibody responses commonly seen with MCV is the induction of T-suppressor cells concomitant with T-helper and B-cell activation. Various immunologic manipulations have been attempted in the past to circumvent this specific suppressor effect with variable success. Cyclophosphamide has been used extensively in this regard and is known to inhibit T-suppressor activation, see for example, Berd, D. et al., Canc. Res. 46:2572 (1986). In this trial, one treatment arm included cyclophosphamide in an effort to combat this very problem. There was no indication that cyclophosphamide influenced the incidence or magnitude of the anti-U-TAA antibody response. However, anti-U-TAA immunity may have been prolonged in the patients receiving this drug.

The antigenic activity of normal urine was completely destroyed by treatment with mixed glycosidases and virtually not at all by proteases. On the contrary, the antigenic activity in melanoma urine was the least susceptible to mixed glycosidase and most susceptible to

proteases and carboxypeptidase. These results clearly indicated that the antigenic cross-reactivity of normal urine was due to carbohydrate portion of the urinary tumor-associated antigenic complex of cancer patients. These
5 carbohydrate moieties are present on molecules in normal urine and on urinary TAA complex in urine of cancer patients. Thus, the invention provides a method of detecting or monitoring a cancer in a subject having a urinary antigenic complex resulting from the cancer
10 comprising, removing a sample from the subject, altering the carbohydrate portion of the urinary antigenic complex in the sample so as to prevent binding with reagents which bind to Urinary Tumor Associated Antigen, and detecting at least a portion of the altered urinary antigenic complex
15 and thereby detecting the cancer. By altering is meant any change which prevents or reduces the number of false positives resulting from the reagent binding with the carbohydrate portion of the urinary antigenic complex. Such alteration could, for example, be accomplished by
20 reducing the carbohydrate portion.

The invention also provides two epitopes of Urinary Tumor Associated Antigen located on the 45 kD and 65 kD polypeptide subunits after reduction by β -mercaptoethanol
25 and separation by SDS-polyacrylamide gel electrophoresis, and reactive with autologous human serum. The invention also provides an epitope of Urinary Tumor Associated Antigen located on the 120 kD polypeptide subunit after reduction by β -mercaptoethanol and separation by SDS-
30 polyacrylamide gel electrophoresis and reactive with baboon polyclonal antibodies. The invention also provides an epitope of Urinary Tumor Associated Antigen located on the 90-100 kD polypeptide subunit after similar reducing and reactive with the murine monoclonal antibody provided for
35 herein. The demonstration of four different epitopes is demonstrated by the lack of cross-reactivity between different types of antibodies as taught herein.

The presence of antigenically distinct epitopes allows for a method of detecting Urinary Tumor Associated Antigen in a sample comprising contacting the Urinary Tumor Associated Antigen with a first reagent which binds to an epitope on Urinary Tumor Associated Antigen, contacting the Urinary Tumor Associated Antigen with a second reagent which binds to a second epitope on Urinary Tumor Associated Antigen, binding one of the reagents to a solid support and detecting the presence of the unbound reagent and thereby detecting the presence of Urinary Tumor Associated Antigen. The reagents can be antibodies and the first antibodies can be monoclonal and the second antibodies can be polyclonal. Further, the reagent can be bound to the solid support prior to binding to an epitope on Urinary Tumor Associated Antigen.

The following examples are intended to illustrate, but not to limit, the invention.

EXAMPLE I

Purification of U-TAA from Urine: U-TAA was prepared from the urine of a melanoma patient with disease metastatic to the liver and spleen. The urine was collected over a twenty-four hour period in 0.1 M Tris (pH 8.3) and 0.02% sodium azide as preservatives. Two liters of urine were concentrated to 20 ml using a hollow fiber concentrator with an exclusion limit of 10,000 daltons (Amicon Corp., Danvers, MA). Seven ml of the concentrate were applied to a 100 x 2 cm Sephacryl S-200 column. PBS was used as the eluent at a flow rate of 14.5 ml/hr, and 3.5 ml fractions were collected. The elution profile was monitored at 280 nm. Fractions were assayed for U-TAA activity using allogeneic antibody in a double determinant EIA. Fractions exhibiting the greatest U-TAA activity were pooled and concentrated to 7 ml by ultrafiltration (PM-20

membrane, Amicon Corp., Lexington, MA). The concentrate was further purified by incubation with 20% (v/v) rabbit antihuman immunoglobulin immunobeads (Bio-Rad Laboratories, Richmond, CA) for 1 hour at 37°C. Protein content of the
5 purified antigen was estimated by the method of Lowry (Lowry, O.H. et al., J. Biol. Chem. 193:265-275 (1951) incorporated by reference herein). Antigen prepared in this fashion was used as a standard or target in immunoassays and as immunogen for production of
10 xenoantibody and murine monoclonal antibody.

The U-TAA activity of the resultant fractions was measured by the allogeneic double determinant EIA. In this assay, anti-U-TAA IgM was used to capture the antigen and
15 allogeneic anti-U-TAA IgG as the detecting antibody. Though comprising only 0.6% of the total protein, the void volume of the Sephacryl S-200 column contained 63% of all of the U-TAA activity detectable in this urine. This represented a 105-fold enrichment of U-TAA (Figure 1).
20 Eighteen percent of the U-TAA was detected in the second peak of the chromatogram, but only the first peak was used as target antigen or immunogen in these investigations. A 24-hour urine collection from this patient typically yielded 0.7 mg of U-TAA.

25

EXAMPLE II

Purification of Human Allogeneic Anti-U-TAA IgM and IgG Antibodies: Initially, IgG and IgM were isolated from
30 the sera of melanoma patients exhibiting high anti-U-TAA antibody levels and were employed in a double determinant EIA for the detection of U-TAA. IgG was isolated by DEAE Affigel Blue chromatography (Bio-Rad Laboratories, Richmond, CA). Ten ml of the serum was dialyzed for 48 hrs
35 against 2 liters of 0.02 M potassium phosphate buffer (pH 8.0) with 4 buffer changes. Following dialysis, the serum was centrifuged at 800 x g for 15 min. and the supernate

passed through a 35 ml bed volume of DEAE-Affigel Blue and eluted with the potassium phosphate buffer. The ~~eluent~~^{eluant} was collected in 10 ml fractions. The fractions demonstrating absorbance of greater than 0.05 at 280 nm were pooled and concentrated to 10 ml. The allogeneic anti-U-TAA IgG level of the concentrate was measured by an enzyme linked-immunosorbent assay (ELISA). The IgG preparation contained 2.23 mg protein/ml and had an anti-U-TAA titer of 1:2560.

Proteins that remained bound to the DEAE Affigel Blue were eluted with 0.02 M phosphate buffer supplemented with 0.5 M NaCl (pH 8.0) and concentrated to 10 ml. The concentrate was subjected to Sephacryl S-300 (Pharmacia Uppsala, Sweden) gel filtration chromatography to enrich anti-U-TAA IgM antibody. The fractions containing anti-U-TAA IgM antibody activity by ELISA were pooled and concentrated. The IgM preparation contained 0.89 mg protein/ml and had an anti-U-TAA titer of 1:150. These antibodies have been used successfully in a capture assay for U-TAA (Euhus, D., et al., The FASEB Journal 2(6):A1836 (1988)). While the isolated anti-U-TAA IgM and IgG antibodies reacted strongly with the U-TAA, they showed minimal or no reactivity with normal urine components.

25

EXAMPLE III

Production of Xenogeneic Anti-U-TAA Serum: Anti-U-TAA xenoantisera were prepared in the baboon and were used in ELISA and immunoblotting for the detection of U-TAA. A 12 year old male baboon weighing 28 kg was injected intramuscularly at predetermined (one to four week) intervals with 100 µg U-TAA mixed with an equal volume of Mylanta (Stuart Pharmaceuticals, Wilmington, DE). The baboon was bled periodically and serum anti-U-TAA levels were measured by ELISA.

After four injections of U-TAA over the course of 6

weeks, the baboon developed detectable anti-U-TAA IgG levels. The antibody titers peaked at the 40th week. At this time the anti-U-TAA antibody titer by ELISA was 1:200,000.

5

IgG and IgM antibodies from the baboon serum were purified by DEAE Affi-Gel Blue chromatography as described above in Example II (Figure 2). IgG antibody was used in the double determinant EIA and IgM for cytotoxicity studies.

10

EXAMPLE IV

Cytotoxic Effect of Baboon Anti-U-TAA Antibodies:

Both IgG and IgM baboon anti-U-TAA antibodies were tested for their cytotoxic effect in the complement dependent cytolysis (CDC) assay using UCLA-SO-M14 as the target. Although the baboon anti-U-TAA IgG antibody was not cytotoxic under the experimental conditions described below, the IgM antibody was cytotoxic.

20

Target cells (tumor and control) were harvested in mid log phase of growth, washed twice with RPMI-10% FCS, and labeled with 51 Cr. The labeled cells were seeded into the cytotoxicity assay plate at a concentration of 1.0×10^4 cells/well in 50 μ l volume. The cells were then mixed with 50 μ l of baboon IgM antibody (30 μ g protein/ml) and incubated at 4°C for 1.0 hr, followed by the addition of 100 μ l baby rabbit complement at 1:10 dilution. The assay plates were further incubated at 37°C for 2 hrs. After centrifugation of the plates (500 X g for 5 min), 100 μ l supernates from each well were aspirated and radioactivity release was assessed by gamma counting. Maximum lysis (total radioactivity added to each well) was determined by adding 150 μ l of 0.05% ~~Nonidet~~ ^{Nonidet} P-40 (NP-40) in PBS. The spontaneous release of the isotope was determined from the supernate of those wells that did not receive the antibody.

35

The percentage of cytolysis was calculated from the mean of triplicate well using the following formula:

$$\% \text{ cytolysis} = \frac{\text{CPM with antibody} - \text{CPM without antibody}}{\text{CPM in maximum lysis}} \times 100$$

The cytolysis of UCLA-SO-M14 cells by 50 μ l of baboon anti-U-TAA IgM antibody at 1:120 dilution (1.5 μ g protein) in CDC was 50 to 60% in contrast to 5% of autologous lymphoblastoid, L14 (normal control) cells. Absorption of the antibody with an increasing number of M14 or L14 cells revealed that, while 1 X 10⁷ L-14 cells did not affect the cytolysis of M14 cells, absorption with M14 cells decreased the cytolysis with increasing numbers of cells (M14). The cytolysis was abolished by absorption with 1 X 10⁷ M14 cells (Figure 3).

To determine whether the inhibition of cytolysis was due to the expression of U-TAA on the surface of M14 cells, the antibody was mixed at various dilutions with an equal volume of purified U-TAA (0.06 mg/ml) and incubated at 37°C for 1 hr and then tested in the CDC assay. Figure 4 illustrates that the cytolysis of M14 cells at 1:120 dilution of the baboon IgM anti-U-TAA was decreased from 35% to less than 5% in the presence of U-TAA. Clearly, purified U-TAA produced the same effect as whole melanoma (M14) cells. Irrelevant proteins, e.g. fetal calf serum (10%), which was included in the assay medium, did not affect the cytolysis.

To determine the cytotoxic effect of baboon IgM anti-U-TAA antibody on cell lines other than M14, various human cultured tumor cell lines, including lymphoblastoid cells, were used as targets in the complement dependent cytotoxicity assay. Tumor cell lines other than melanoma, e.g. breast carcinoma and neuroblastoma, were also lysed by the baboon IgM anti-U-TAA antibody (Table 1). The

cytolysis of lymphoblastoid cell line (L 15) was not higher than the background (>5%). These results denote that U-TAA is expressed on the surface of a variety of melanoma and non-melanoma tumor cells and that its density varies from cell line to cell line.

Table 1

Cytotoxicity of baboon IgM anti-U-TAA antibody in CDC assay to different target cell lines. (Ten thousand chromium labeled cells of each cell line were reacted with 50 μ l [1.0 μ g protein] of the antibody in the presence of rabbit complement. Cytolysis was assessed by the release of radioactivity.

<u>Cell line</u>	<u>Type</u>	<u>%Cytolysis \pm SD</u>
L15	Lymphoblastoid (normal control)	4.9 \pm 4.9
M9	Melanoma	40.0 \pm 5.0
M10	"	71.4 \pm 0.4
M14	"	68.3 \pm 5.6
M16	"	20.8 \pm 4.0
M24	"	36.5 \pm 2.8
M101	"	25.3 \pm 4.6
M109	"	29.0 \pm 2.7
CPR	Breast carcinoma	36.5 \pm 9.1
MCF	"	15.0 \pm 6.2
CHP	Neuroblastoma	35.5 \pm 1.5

EXAMPLE V

Murine Hybridoma Technique: An eight week old brown female C57BL/6 mouse was injected intraperitoneally (ip) with 75 μ g of U-TAA (Ne8704) in PBS on days 0, 15, and 28. On day 37, the mouse was boosted with 150 μ g U-TAA ip and sacrificed three days later to obtain hyperimmune spleen cells. The cells were fused with 8-azaguanine resistant, non-secreting mouse Sp 2/0 myeloma cells in a manner similar to that described by Galfre (Galfre, G. et al., Nature 277:131 (1979) incorporated by reference herein). Hybridoma cells were seeded onto plates containing 3 day old mouse peritoneal macrophage cultures obtained by rinsing the peritoneum of a C57BL/6 mouse with 5 ml of 11.6% sucrose and plating the macrophages at 1.45×10^4 cells/well.

Supernatants from wells containing healthy colonies were screened for anti-U-TAA antibody in ELISA. Positive wells were cloned using the method of limiting dilutions. Monoclonal antibody was prepared from mouse ascites (Hoogenraad, N. et al., J. Immunol. Methods 61:317-320 (1983) incorporated by reference herein). Antibody isotypes were determined by double immunodiffusion (Miles Laboratories, Naperville, IL).

EXAMPLE VI

Target Antigens for Hybridoma Supernate Screenings: Hybridoma supernatants were tested in ELISA for reactivity with various U-TAA preparations and normal urines. U-TAA was purified from urine samples of three different melanoma patients, Ne8704, Wo7907, and Se8703, by Sephacryl S-200 gel filtration chromatography. Ten normal urines were used as target antigen after 100-fold concentration. Each target was used at 1.4 μ g protein/ml. In each case antigen was diluted in 0.06 M sodium carbonate buffer (pH 9.6) and

immobilized on microtiter plates by incubation for 3 hr at 37°C. This and each subsequent incubation was followed by three washes in PBS supplemented with 0.05% Tween-20 (PBS-T). Alkaline phosphatase conjugated to goat anti-mouse Ig
5 (Jackson ImmunoResearch, West Grove, PA) provided the catalyst for conversion of the non-chromogen, P-nitrophenyl phosphate (1 mg/ml in 10% diethanolamine, pH 9.6), to chromogen, P-nitrophenyl. The absorbance of each well was measured at 405 nm using the multiscan ELISA plate reader.

10

During the course of immunization the anti-U-TAA antibody activity in the mouse serum increased from undetectable to 1:8000 against Ne8704 and 1:7730 against Wo7907 by ELISA. All of the 96 wells seeded with fusion
15 products of the immunized mouse spleen cells grew healthy hybridomas. Supernatants of 51 of these hybrids contained antibody reactive with Ne8704, but only 13 were positive against both Ne8704 and Wo7907. Cells from these 13 wells were cloned. Among the resultant clones, only one
20 designated as AD1-40F4 produced an antibody that reacted with U-TAA of Ne8704, Wo7907, and Se8703 urine samples but not with two concentrated normal urines. AD1-40F4 proved to be an IgM molecule by double immunodiffusion. Unprocessed hybridoma supernatant of this clone had an
25 anti-U-TAA IgM titer of 1:200. AD1-40F4 raised in mouse ascites had an anti-U-TAA IgM titer of 1:2000-1:5000 and was used in subsequent experiments at 1:200-1:500 dilutions.

30

EXAMPLE VII

Specificity Analysis of AD1-40F4: A murine monoclonal antibody capable of differentiating between U-TAA and normal urine was raised in mouse ascites and tested for
35 reactivity with a variety of immobilized targets (Euhus, D. et al., J. Clin. Lab. Anal. 3:184 (1989)). The ascite was used at 2 µg/ml in all of the following assays. To assess

whether the antibody recognized some common human or fetal calf serum protein, the following commercially available proteins were used at 10 µg/ml as targets in ELISA: ferritin, pooled human IgG, pooled human IgM (Cooper
5 Biomedical, Westchester, PA), B2-microglobulin, B2-glycoprotein, apolipoprotein B, apolipoprotein CII, and apolipoprotein CIII (Calbiochem, La Jolla, CA), human serum albumin (Miles Laboratories, Inc., Naperville, IL), and fetal calf serum diluted 1:10. In addition, 20 normal and
10 52 stage II and III melanoma sera were diluted 1:10, immobilized on polystyrene plates, and then reacted with the antibody. To determine whether the reactivity of the monoclonal antibody correlated with human allogeneic antibody reactivity, 5 melanoma urines positive for U-TAA,
15 5 melanoma urines negative in the allogeneic double determinant ELISA, and 10 concentrated normal urines were used as targets in ELISA.

Murine monoclonal antibody AD1-40F4 exhibited no
20 reactivity with ferritin, pooled human IgG, pooled human IgM, B2-microglobulin, B2-glycoprotein, apolipoprotein B, apolipoprotein CII, apolipoprotein CIII, or human serum albumin. In addition, it did not react with whole fetal calf serum.

25

Monoclonal antibody AD1-40F4 reacted with 65% (33/52) of randomly selected melanoma sera from stage II and III patients who were alive with disease at the time of serum sampling but with only 5% (1/20) normal sera.

30

Five concentrated urines from melanoma patients testing positive for U-TAA in a human allogeneic double determinant ELISA also demonstrated strong reactivity with AD1-40F4, while five urines that were known to be U-TAA
35 negative failed to react with AD1-40F4.

UCLA-SO-M14 is a cultured melanoma cell line that

grows well in chemically defined medium. Concentrated spent media fractionated on a sepharose 6B column was tested in ELISA as the target for murine monoclonal antibody AD1-40F4. The material in the void volume of the
5 column reacted at a dilution of 1:23,000 with AD1-40F4. This dilution corresponded to a protein concentration of 177 ng/ml.

EXAMPLE VIII

10

ELISA: Target antigen or capturing antibody was diluted in 0.06 M sodium carbonate buffer (pH 9.6) and bound to polystyrene microtiter plates (Immulon I, Dynatech Laboratories, Inc., Alexandria, VA) by incubation at 37° C
15 for 2 hr in 100 µl/well aliquotes. The plates were then washed three times with PBS supplemented with 0.05% Tween-20 (PBS-T). Each subsequent reagent was diluted in PBS-T and added in 100 µl aliquotes per well. Each reagent addition was followed by a 45 minute incubation at 37°C and
20 three PBS-T washes. As the final step, 200 µl of para-nitrophenyl phosphate at 1 mg/ml in 10% diethanolamine (pH 9.8) as substrate for the enzyme was added to each well. The plates were incubated at 23°C and color development in each well was measured as absorbance at 405 nm using a
25 Multiscan EIA plate reader (Flow Laboratories, Inc., McClean, VA). Each assay was run in quadruplicate with positive and negative controls, as well as controls for non-specific protein binding and conjugate binding to the immobilized antigen or antibody.

30

EXAMPLE IX

Double Determinant EIA: Initially, the U-TAA content of fractionated melanoma urine was measured using a double
35 determinant EIA, which employed allogeneic anti-U-TAA IgM and IgG antibodies from melanoma patients. In most subsequent experiments, U-TAA content was measured in a

xenogeneic double determinant EIA employing murine monoclonal antibody AD1-40F4 and baboon polyclonal anti-U-TAA IgG.

5 In the allogeneic antibody assay, microtiter plates were sensitized with IgM (allogeneic) antibody at 20 μ g/ml. Serially diluted standards and urine fractions diluted 1:10 in PBS-T were then applied. Allogeneic IgG antibody at 20 μ g/ml in PBS-T was added as the second antibody. Alkaline
10 phosphatase conjugated to goat anti-human IgG (Sigma Chemical Co., St. Louis, MO) diluted 1:1000 in PBS-T was used as the enzyme conjugate. The absorbance at 405 nm was recorded after a 2 hr incubation with the substrate. The U-TAA concentration of each sample was obtained by
15 interpolation from the standard curve. The assay has a sensitivity of 570 ng/ml.

 In the xenogeneic double determinant EIA murine monoclonal antibody AD1-40F4 at 176.0 μ g/ml was used to
20 capture the antigen, while baboon IgG at 8.9 μ g/ml was used as the second antibody. This assay has a sensitivity of 50 ng/ml.

EXAMPLE X

25

Sera for U-TAA Activity: Blood was collected by venipuncture from 52 melanoma patients with disease metastatic to regional lymph nodes, in transit lymphatics, or distant visceral tissues and allowed to clot at room
30 temperature for 2 hrs. Sera was separated from the clotted blood by centrifugation at 800 x g for 15 minutes and immediately frozen at -35°C. Sera from 20 apparently healthy controls was obtained and processed in a similar manner. On the day of assay, the serum samples were
35 thawed, diluted 1:10 in 0.06 M carbonate buffer, and applied to microtiter plates in 100 μ l aliquotes and incubated at 37°C for 2 hrs. After washing, the wells were

reacted with 100 μ l of 1:500 dilution of AD1-40F4 MoAb. Bound AD1-40F4 was labeled with goat anti-mouse immunoglobulin conjugated to alkaline phosphatase (Jackson Immunoresearch, West Grove, PA) that was diluted 1:5000 in
5 PBS-T supplemented with 1% bovine serum albumin (Sigma Chemical Co., St. Louis, MO). Following a 16 hour substrate incubation period, the average absorbance at 405 nm for each sample was determined and corrected for background (binding of AD1-40F4 to plastic and binding of
10 the enzyme conjugate to the target sera). Sixty-three percent (33/54) of disease bearing melanoma patients exhibited reactivity with AD1-40F4. In contrast, only 5% (1/20) of sera from apparently healthy volunteers exhibited the reactivity. However, the reactivity of this normal
15 serum was minimal.

EXAMPLE XI

Isolation of U-TAA from Serum: A variety of
20 chromatographic and affinity absorption techniques were employed to isolate and characterize U-TAA from the positive serum. Special attention was given to the removal of IgG from the antigen preparation to ensure that AD1-40F4 was recognizing a novel macromolecule and not an
25 idiotypic determinant on an antibody molecule.

Selected serum samples were applied to a DEAE-sephacel column and eluted at a flow rate of 30 ml/hour with a pH (8 to 5) and salt (0.015 to 0.3M) gradient formed by slowly
30 siphoning 0.3 M KH_2PO_4 (pH 5.0) into a vessel containing 200 ml of 0.015 M K_2HPO_4 (pH 8.0). Two milliliter fractions were collected and the absorbance at 280 nm and pH of each fraction were determined. The U-TAA activity of
35 fractionated sera was measured using the xenogeneic double determinant EIA described above. Fractions containing the U-TAA activity were pooled and concentrated to 2 ml by ultrafiltration (Figure 5).

The majority of serum IgG eluted in the first peak of the column; however, small amounts were present in the second peak as well. As expected, IgM was detected only in the last peak. The elution profile of a normal serum was similar to that of a melanoma serum, but no U-TAA was detectable in any fraction. In the U-TAA positive melanoma sera, the antigen was eluted primarily with the second peak, though small amounts were eluted with IgG in the void volume and traces were detected in association with IgM. U-TAA in the second peak eluted at pH 7.27, which corresponds to a K_2HPO_4 molarity of 0.021. The anion exchange elution profile of the normal serum that was reactive with AD1-4OF4 murine MoAb was identical to that of the serum from melanoma patients, except that no antigen was detected in association with IgM.

Fractions comprising the second peak of the anion exchange column were pooled and concentrated to 2 ml. The protein content of this preparation averaged 3.78 mg/ml. Because this fraction contained small amounts of IgG, it was absorbed sequentially with rabbit anti-human IgG immunobeads to remove the contaminating IgG. One absorption diminished the IgG titer by 95%, while four absorptions removed 99.5% of the IgG. The U-TAA titer, in contrast, was minimally affected.

U-TAA isolated by anion exchange chromatography and absorbed against anti-human IgG immunobeads was further purified by gel filtration chromatography. The sephacryl S-300 chromatogram consisted of one major protein peak and several minor peaks. The U-TAA activity of antigen positive preparations was consistently confined to a symmetrical high molecular weight peak that was distinct from the major protein peak (this peak was not present in preparations from U-TAA negative sera).

EXAMPLE XIISDS-polyacrylamide Gel Electrophoresis (SDS-PAGE):

Fifty μ l of U-TAA from serum at 64 μ g/ml was heated to
5 100°C for one minute in 25 μ l of 0.06 M Tris-HCl (pH 6.8)
containing 12.5% glycerol, 1.3% sodium dodecyl sulfate and
1.3% 2-mercaptoethanol. The reduced sample was loaded onto
a stacking gel containing 5% polyacrylamide and separated
on a 9% polyacrylamide gel at 50 mA for 4 hrs. The running
10 buffer consisted of 0.05 M Tris, 0.38 M glycine, and 0.1%
sodium dodecyl sulfate (pH 8.4). All gels were run in
duplicate. One gel was stained with silver reagent (Bio-
Rad Laboratories, Richmond, CA) and the other was subjected
to immunoblotting.

15

EXAMPLE XIII

Immunoblotting: One of the two electrophoresed
polyacrylamide gels was washed for 30 min with 0.02 M Tris,
20 0.15 M glycine and 20% ethanol and electroblotted to
nitrocellulose paper (0.45 μ m, Bio-Rad Laboratories) at 50
Volts for 16 hrs (4°C). After electroblotting, the
nitrocellulose paper was treated with 5% casein in blotting
buffer (0.05 M Tris, 0.09 M NaCl, pH 8.0) for 2 hrs at
25 23°C. The treated paper was reacted with AD1-4OF4 (1:500)
murine MoAb or baboon anti-U-TAA IgG (1:500) in the
blotting buffer supplemented with 5% casein for 6 hrs at
23°C and then 16 hrs at 4°C. Following incubation with
anti-U-TAA antibody, the paper was washed four times in 200
30 ml blotting buffer. Alkaline phosphatase conjugated to
goat anti-mouse Ig or goat anti-human IgG in blotting
buffer supplemented with 5% casein was used to detect bound
anti-U-TAA antibody. After incubation with the enzyme
conjugate at 23°C for 45 min, the nitrocellulose paper was
35 washed four times with the blotting buffer. After washing
again for one minute in substrate buffer (10mM Tris, 0.1 M
NaCl and 0.05 M $MgCl_2$, pH 9.5), the paper was reacted with

Nitroblue Tetrazolium Chloride/5-Bromo-4-chloro-3 indolyl phosphate p-toluidine (NBT/BCIP) according to the manufacturer's directions (Bethesda Research Laboratories, Gaithersburg, MD). The molecular weights of the positive
5 bands were determined based on the relative migration of prestained molecular weight markers (Bio-Rad Laboratories, Richmond CA).

EXAMPLE XIV

10

SDS-PAGE Analysis of Serum U-TAA: U-TAA from serum separated by SDS-PAGE and stained with the silver reagent produced four bands in the regions of 138, 90, 50, and 25 kD. When transferred to nitrocellulose paper and
15 immunostained, the 90 kD band was the only one which reacted with both murine monoclonal antibody, AD1-40F4, and baboon polyclonal anti-U-TAA IgG (Figure 6). U-TAA from the one cross-reacting normal serum exhibited an identical immuno- staining pattern; however, the similar preparation
20 from U-TAA negative normal sera showed no reactivity with either antibody.

EXAMPLE XV

25 Presence of U-TAA Subunits on Tumor Cells: To determine the expression of ^{the} 90 kD component which is recognized by allogeneic antibody, cell free NP-40 extract of biosynthetically labeled (¹⁴C-L-leucine) melanoma (UCLA-SO-M14) cells was subjected to immunoprecipitation with the
30 allogeneic antibody before and after blocking with U-TAA. The U-TAA (blocker) was purified from the urine of melanoma patients as described in Example I. The NP-40 extract of melanoma cells was prepared as described in Example XXXII. The immunoprecipitates were subjected to SDS-PAGE (12% polyacrylamide). Radioactivity in blocked and unblocked
35 immuno-precipitate lanes of the gel was assessed by scintillation counting. As depicted in Figure 7, three

bands, 150 kD, 90 kD and 45 kD, were partially or completely blocked by U-TAA. These results document that the allogenic antibody reacted with multiple antigenic components of melanoma cells. One of these was a 90 kD subunit that is also present in the urine and serum of melanoma patients and is recognized by the murine monoclonal antibody AD1-40F4.

EXAMPLE XVI

10

Isoelectric Focusing: Isoelectric focusing of serum U-TAA was performed in a 5% polyacrylamide gel using an LKB 2117 Multiphor system (LKB Instruments, Inc., Rockville, MD). A strip of filter paper was soaked in 25 μ l of U-TAA and placed 1 cm from the cathode edge on the PAG plate (LKB Instruments, Inc.), which contained 2.4% (w/v) ampholine solution with a pH gradient of 3.5-9.5. H_3PO_4 (1 M) and NaOH (1 M) were used at the anode and cathode, respectively. The plate was electrofocused by application of 1500 volts, 50 mA, and 30 watts for 1.5 hours at 10°C. Commercially available chromogenic proteins of known isoelectric point (Pharmacia Fine Chemicals, Piscataway, NJ) were included in each run. A portion of the gel was cut and stained with coomassie blue. However, because the protein content of the U-TAA preparations was quite low (64 μ g/ml) coomassie blue staining did not visualize the U-TAA bands. To overcome this difficulty the remainder of the gel was analyzed by the Western blot technique.

30

Focused gels were blotted onto nitrocellulose and reacted with baboon anti-U-TAA IgG. Only one band was visualized by this technique, making it possible to assign an isoelectric point of 6.1 to U-TAA purified from the serum (Figure 8).

35

EXAMPLE XVII

Heat Stability: To assess the heat stability of the immunoreactive epitope, U-TAA preparation was heated to 100°C for 15 min and for one hr. This preparation, as well as the corresponding unheated preparation, was used at 1.4 $\mu\text{g/ml}$ as target for the antibody.

Heating the solution to 100°C for up to 1.0 hr did not affect the antigenic activity significantly (Table 2).

10

TABLE 2

Heat Stability of U-TAA Isolated From
Serum of a Melanoma Patient

15

<u>Treatment</u>	Absorbance at 405 nm in Xenogeneic <u>Double Determinant EIA</u>
Control ^a	0.635 \pm 0.035 ^b
100 °C for 15 min	0.578 \pm 0.042
100°C for 1 hr	0.594 \pm 0.065

20

^a. U-TAA was heat treated at a protein concentration of 200 $\mu\text{g/ml}$ and used in the assay at 1.4 $\mu\text{g/ml}$ as the target antigen.

^b. Mean \pm standard deviation of triplicate samples.

25

EXAMPLE XVIII

Enzyme Digestions: To assess the effects of glycosidase digestion on the U-TAA activity of normal and melanoma urines as determined by the allogeneic double determinant ELISA, mixed glycosidase (Miles Scientific, Naperville, IL) were coupled to CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) according to the manufacturer's specifications. The activity of the immobilized glycosidase was assessed by recording hydrolysis of a standard p-nitrophenyl-2-D-glucuronide solution. Twenty-four hour urine specimens were collected from 14 individuals (7 apparently healthy, 7 stage II melanoma alive with disease) in 0.1 M Tris (pH 8.3) supplemented with 0.1% sodium azide. Initial volume and creatinine content (Beckman Creatinine Analyzer, Fullerton, CA) were determined for each specimen prior to 100-fold concentration in a recirculating hollow fiber concentrator with an exclusion limit of 10,000 daltons (Amicon Corp., Lexington, MA). Concentrated urines were treated with enzyme charged gel (or unlabeled gel prepared in an identical fashion and blocked with 1M ethanolamine) for 4 hrs at 37°C. Treated and untreated urines were then tested for U-TAA activity in the allogeneic double determinant ELISA described above. Results (U-TAA ng/100 ml) were converted to antigen units (Ag U)/mg creatinine to compensate for differences in initial urine volume and interassay variation as follows:

$$\text{AgU/mg creat} = \frac{[\text{U-TAA}] \times F \times 100}{[\text{creat}] \times K}$$

where Ag U/mg creat is antigen units per mg creatinine, [U-TAA] is U-TAA concentration of the sample in ng/ml interpolated from the standard curve. F is the final volume of the 100-fold concentrated urine sample; [creat] is creatinine content (in mg) of the entire 24-hr urine sample and K is the protein concentration (ng/ml) of the U-

TAA standard producing an O.D. at 405nm of 1.0 in this assay. This concentration is arbitrarily assigned a value of 100 antigen units.

5 Urine samples from seven apparently healthy donors had a mean U-TAA content of 21.9 ± 4.0 antigen units/mg creatinine (by alloantibody assay), while sample from seven stage II melanoma patients alive with disease had a mean U-TAA content of 69.8 ± 17.4 antigen units/mg creatinine
10 ($P < 0.025$). Mixed glycosidase treatment of specimens from normal donors lowered the mean antigen level slightly to 14.8 ± 2.8 $P > 0.1$, while similar treatment of melanoma urines significantly augmented these antigen levels to 163.7 ± 49.4 , $P < 0.025$.

15

EXAMPLE XIX

Characterization of Murine Monoclonal Antibody (AD1-40F4) Reactive Epitope: To elucidate the chemical nature
20 of the AD1-40F4-reactive epitope, U-TAA was degraded with various enzymes immobilized on agarose beads. Mixed glycosidase (Miles Scientific) and hyaluronidase (Worthington Biochemical, Freehold, NJ) were coupled to CNBr activated agarose beads (Sephacrose 4B, Pharmacia Fine
25 Chemicals, Uppsala, Sweden). The immobilized protease was purchased from Sigma Chemical Co. (St. Louis, MO). The specific activity of each immobilized enzyme was tested against the appropriate substrate system before treatment of the antigen.

30

U-TAA at $36 \mu\text{g/ml}$ was continuously mixed end-over-end for 4 hrs at room temperature with an equal volume of packed-immobilized enzyme beads. CNBr activated agarose beads (without enzyme) blocked with 1M ethanolamine, were
35 used as untreated control. U-TAA activity of the enzyme treated and untreated supernatants was assessed in a competitive inhibition assay as follows: Treated and

untreated U-TAA were diluted to 0.63 $\mu\text{g/ml}$ and incubated for 45 min at 37°C with AD1-40F4 murine monoclonal antibody ascites diluted at 1:375. Binding of the AD1-40F4 in this mixture to immobilized U-TAA was then quantitated in an ELISA. Following correction for non-specific binding to plastic and target antigen, the absorbance at 405 nm was converted to percent inhibition. Each assay was run in triplicate and an average percent inhibition \pm SEM was calculated. The inhibitory activity of enzyme treated antigen was compared with that of untreated antigen in a simple ratio (% inhibition treated / % inhibition untreated). These ratios provided a basis for comparing the effects of the three enzymes on U-TAA.

One would expect that treatment of U-TAA with an enzyme that does not affect the immunoreactive epitope would result in an activity ratio near 1.0:1. This was the case with hyaluronidase treatment, where the activity ratio of treated to untreated antigen was 1.5:1. The treatment with glycosidase enhanced the antigen activity (ratio of 3.2:1), while protease treatment significantly diminished the U-TAA activity resulting in a ratio of 0.2:1. Prolonging the protease incubation time by 8 hrs destroys all U-TAA activity.

25

EXAMPLE XX

Patients: Serum samples from 15 patients undergoing active specific immunotherapy with the MCV were collected prior to immunization and every 2 to 4 weeks thereafter for analysis of anti-U-TAA antibody levels. Seven of the 15 patients evaluated had received cyclophosphamide with their MCV. Samples were also collected from 7 unvaccinated Stage II and III melanoma patients for comparison. The samples were stored at -40°C, and all samples for a given patient were thawed and analyzed on the same day. To ensure that at least half of the subjects were capable of mounting an

antibody response to cell membrane antigens, patients were chosen on the basis of their antibody response to M-TAA; eight of the 15 patients had demonstrated induction of anti-M-TAA antibodies during the course of vaccination, while 7 did not. This also allowed us to determine whether antibody response to one membrane antigen guaranteed antibody response to other antigens on the same cell surface.

Vaccination Protocol: First vaccination comprised of 24 million live, irradiated tumor cells (from cell lines designated UCLA-SO-M10, UCLA-SO-M24 and UCLA-SO-M101 which were selected for their antigenic variability) mixed with 24 million Glaxo BCG organisms. Second vaccination consisted of a mixture of 24 million live, irradiated tumor cells and 12 million Glaxo BCG organisms. Subsequent vaccinations consisted of 24 million live and irradiated tumor cells without BCG. The immunization schedule consisted of biweekly vaccinations for the first six weeks and every four weeks thereafter.

Statistical Analysis: Antibody titers in various groups are expressed as means \pm standard error of the mean (SEM). Relevant comparisons were made using students' paired T-tests and Chisquare analysis. The statistical software employed in these analyses was StatView (Brain Power, Inc., Calabasas, CA) for the Macintosh computer.

EXAMPLE XXI

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Determination of Serum Anti-U-TAA Antibody Titers: Forty nanograms of U-TAA in 100 μ l of 0.06M carbonate buffer (pH 9.6) was dispensed into 96-well polystyrene microtiter plates. Following a 3 hour incubation at 37°C, the wells were washed 3 times in PBS-T, and 100 μ l of serially diluted serum was applied. After a 45 minute incubation at 37°C, the wells were washed and anti-U-TAA

antibody was detected using alkaline phosphatase conjugated to goat anti-human IgG (diluted 1:500) or IgM (diluted 1:1000). The plates were incubated for 45 minutes and washed 3 times in PBS-T. Substrate was applied and after
 5 a one-hour incubation at 23°C, the O.D. _{405nm} was determined for each well. All assays were run in duplicate with appropriate controls for non-specific binding of the conjugate to plastic and target antigen. Titration curves were generated by plotting the average corrected O.D. _{405nm}
 10 for the duplicate samples against the serum dilution (\log_2). The linear portion of the titration curve was extrapolated and the serum dilution corresponding to an O.D. of 0.05 was designated as the anti-U-TAA "titer" of that particular sample.

15

EXAMPLE XXII

Anti-U-TAA IgM Antibody Response in Melanoma Patients:

There was no significant difference in the anti-U-TAA IgM
 20 titers in non-vaccinated melanoma control patients and MCV patients prior to receiving the vaccine ($1:1324 \pm 254$, $N=7$, $1:1138 \pm 198$, $N=15$, $P>0.375$). However, these levels were significantly lower in a group of 8 apparently healthy donors. Pre-vaccination anti-U-TAA IgM titers ranged from
 25 $1:112$ to $1:2466$ in the 15 patients who received the MCV (Table 3). Eleven of the 15 patients receiving MCV demonstrated a 2 to 5-fold increase in these titers during the course of vaccination. Mean IgM anti-U-TAA titers for these 11 patients rose from $1:1051 \pm 259$ pre-vaccination to
 30 $1:2518 \pm 576$ post-vaccination ($P<0.005$). Individual peak anti-U-TAA IgM titers ranged from $1:435$ to $1:5140$.

EXAMPLE XXIII

35 Anti-U-TAA IgG Antibody Response: Pre-vaccination anti-U-TAA IgG titers in the 15 MCV patients ranged from $1:1062$ to $1:8361$ (Table 3). The mean pre-vaccination titer

of 1:3984 \pm 602 in this group of patients is higher than that of the 7 unvaccinated melanoma control patients (1:2595 \pm 423, $P < 0.1$). The mean anti-U-TAA level for 8 apparently healthy controls was significantly lower than
5 that of the unvaccinated melanoma control group. Six of the 15 patients receiving the MCV demonstrated a 2 to 7-fold rise in anti-U-TAA IgG titers. Mean anti-U-TAA IgG titers in this group of 6 responders rose from 1:2964 \pm 1046 pre-vaccination to 1:9958 \pm 2677 post vaccination
10 ($P < 0.01$). Individual peak titers in this group ranged from 1:2,176 to 1:20,481.

TABLE 3

Anti-U-TAA IgM and IgG responses in 15 patients receiving melanoma cell vaccine (MCV)

PATIENT	^a CTX	^b M-TAA	IgM			IgG			
			^c PRE-MCV	^d POST-MCV	^e WEEKS TO RESPONSE	^f RESPONSE DURATION	PRE-MCV	POST-MCV	WEEKS TO RESPONSE
1	+	-	1,200	1,049	-	-	3,200	4,045	-
2	+	-	877	736	-	-	2,445	3,635	-
3	-	+	1,696	3,529	2	4	5,056	7,270	-
4	+	-	1,011	2,445	8	4	1,587	1,869	-
5	+	-	1,562	1,421	-	-	7,987	20,481	>10
6	+	-	112	515	12	>8	4,915	5,990	-
7	+	+	218	448	8	>8	5,348	3,599	-
8	+	+	1,466	5,140	4	4	2,274	14,848	>36
9	-	-	1,411	5,043	20	>20	2,816	7,578	>4
10	-	+	464	1,261	6	4	1,062	2,176	4
11	-	+	1,866	1,933	-	-	2,496	7,680	>4
12	-	-	134	435	12	4	8,361	8,456	-
13	-	+	2,278	4,992	16	4	1,149	6,982	>40
14	-	+	384	1,152	4	>20	6,066	6,651	-
15	-	+	1,389	2,742	16	8	4,992	6,349	-

^aMCV along (-) or with cyclophosphamide (+); ^bpatient exhibiting good anti-M-TAA response (+);

^creciprocal mean prevaccination antibody titer; ^dreciprocal post-vaccination peak titer;

^eweeks for MCV to induce >2-fold rise in antibody titer; ^fnumber of weeks in which >2-fold elevation in antibody titer persisted.

EXAMPLE XXIV

HLA Studies: To assess whether anti-HLA antibodies could account for part or all of our observed antibody responses, the following experiments were performed. First, to mitigate the possibility that our U-TAA preparation was contaminated with HLA, U-TAA was immobilized on polystyrene microtiter plates and tested for reactivity with a murine monoclonal anti-HLA antibody in an EIA (AXL 859M, Accurate Chemical and Scientific Corp., Westbury, NY). This antibody is directed against a monomorphic epitope which occurs on the 45 kD polypeptide products of the Class 1 HLA. Thus, any Class 1 subtypes present in the U-TAA preparation would be detected. A murine monoclonal anti-U-TAA antibody diluted 1:750 was run simultaneously as a positive control.

Secondly, as part of a study to determine the relationship between induction of anti-tumor antibodies and anti-HLA antibodies, 8 pre- and postvaccination sera of melanoma patients who received a melanoma cell vaccine (MCV) were tested for anti-HLA antibodies in a cytotoxic antibody assay that quantitates reactivity with the individual Class 1 and DR antigens (Terasaki, P.I. et al., Manual of Tissue Typing Techniques, DHEW Publ. (NIH) 74-545 U.S. Government Printing Office, Washington, DC, p. 54, incorporated by reference herein). These pre- and postvaccination reactivities were then compared with anti-U-TAA antibody levels.

The murine monoclonal antibody directed at a monomorphic epitope of the HLA Class 1 antigens failed to react with our U-TAA preparation even at dilutions ranging from 1:5 to 1:80. An anti-U-TAA murine monoclonal antibody run simultaneously at a dilution of 1:750 developed intense

color within 1 hour.

In addition, development of anti-HLA antibodies did not correlate in any way with anti-U-TAA antibody response. Only one of the 8 patients tested developed a strong anti-HLA antibody response during the course of vaccination. Before vaccination, this patient had undetectable anti-HLA antibody levels but high anti-U-TAA IgG levels (1:5056). During the course of vaccination he developed high levels of antibody to a variety of Class I HLA's but no change in his anti-U-TAA IgG level. The anti-U-TAA IgM titer merely doubled during this period. Another patient, number 8, never developed significant anti-HLA levels, but his anti-U-TAA IgG and IgM titers rose 6.5-fold and 3.5 fold respectively during the course of vaccination. Four other patients exhibited 2 to 4-fold increases in their anti-U-TAA IgM levels without evidencing induction of anti-HLA antibodies.

20

EXAMPLE XXV

Anti-KLH IgG Antibody Response: To determine whether or not the antibody response to MCV detected in melanoma patients by ELISA was specific to U-TAA, the same serum samples (pre- and post-MCV) were reacted against KLH as the target antigen. Results presented in Table 4 denote that the majority of the patients (14/15) had detectable levels of anti-KLH antibodies before MCV treatment. These antibody levels increased by greater than 2-fold in only two patients after administration of MCV. In other patients, the anti-KLH levels either remained more or less the same or decreased. There was no concordance between the increase in anti-KLH and the anti-U-TAA antibodies (IgG or IgM) in response to MCV.

35

TABLE 4

5 Anti-KLH Antibody Levels in Melanoma Patients Before and After MCV Treatment as Assessed by ELISA

10	Patient #	Anti-KLH titer ^a		Weeks Post-MCV
		Pre-MCV	Post-MCV	
	1	400	480	4
	2	460	62	4
	3	<20	4,000	2
15	4	68	20	8
	5	260	940	4
	6	150	105	12
	7	60	600	8
	8	580	780	4
20	9	400	190	20
	10	280	850	6
	11	290	250	4
	12	1,000	1,050	12
	13	140	240	16
25	14	330	110	4
	15	125	96	16

^a Reciprocal of the titer was determined from the point on the serum dilution curve that exhibited 0.05 O.D. 405nm

30

EXAMPLE XXVI

Time Course of Antibody Response: Four of the 11
 35 anti-U-TAA IgM responders developed ≥2-fold elevations in these antibody levels 2 to 6 weeks following the first vaccination, 6 after 7 to 16 weeks, and 1 after 20 weeks of immunization. Median time to anti-U-TAA IgM response was 8 weeks. Anti-U-TAA IgM titers remained elevated from 4 to
 40 >20 weeks with a median of 8 weeks in these 11 patients.

Three of the 6 patients mounting good anti-U-TAA IgG responses demonstrated ≥2-fold elevations in these titers 4 to 8 weeks following the first vaccination, one responded
 45 after 20 weeks and 2 required 40 weeks of vaccination to

produce IgG responses. Median time to response was 14 weeks. The responses in these 6 patients lasted from 4 to > 40 weeks, with a median of > 10 weeks.

5

EXAMPLE XXVII

Stage of Disease and Prior Anti-M-TAA Response: Of the 15 patients evaluated in this study, 11 had disease confined to the lymph nodes or locoregional subcutaneous tissues, while 4 had distant metastases. All 4 patients with visceral metastases (3 with pulmonary metastases and 1 with a liver metastasis) mounted good anti-U-TAA IgG responses. Prior to vaccination, these anti-U-TAA IgG levels ranged from 1:1149 to 1:2816 (mean 1:2184 \pm 362). Postvaccination the titers peaked at 1:5000 to 1:14,848 with a mean of 1:8777 \pm 2117, a 4-fold increase for the group.

Anti-melanoma-TAA antibody levels from sequential serum samples were available for all 15 patients evaluated in this study (Gupta, R.K. et al., Proc. Amer. Soc. Clin. Oncol. 6:249 (1987) incorporated by reference herein). Eight of these 15 patients had mounted good anti-M-TAA antibody responses with MCV. Augmentation of anti-melanoma-TAA antibody levels did not correlate with augmentation of anti-U-TAA IgG levels ($R^2=0.279$). Overall, the anti-U-TAA response rate (IgM and/or IgG) is higher for M-TAA responders than for M-TAA non-responders, but the difference is not significantly different (X^2 , $P > 0.3$).

30

EXAMPLE XXVIII

Detection of U-TAA in the Urine of Patients with Breast, Colon and Lung Carcinomas: Urine samples obtained from patients suffering from carcinoma of the breast, colon

35

or lung were analyzed for the presence of U-TAA using the double determinant EIA that employed murine monoclonal AD1-40F4 and baboon polyclonal anti-U-TAA IgG as described in Example IX. The incidence of the presence of detectable levels of U-TAA is listed in Table 5. These incidences are quite comparable with that of melanoma patients (63.4%).

Table 5

Incidence of U-TAA in urine of patients with carcinoma of the breast, colon and lung by the double determinant EIA.

<u>Histologic Type</u>	<u># Tested</u>	<u># Positive</u>	<u>% Positive</u>
Breast Carcinoma	14	9	64.3
Colon Carcinoma	13	9	69.2
Lung Carcinoma	7	3	42.8
Sarcoma	17	10	58.8
Normal	77	2	2.6
Melanoma	115	73	63.4

*Murine monoclonal antibody AD1-40F4 was used to capture the antigen and baboon polyclonal antibody to detect the captured antigen as described in Example IX. An EIA value of greater than 0.68 was considered positive.

EXAMPLE XXIX

Monitoring of Malignancy Using U-TAA Levels in Urine of Cancer Patients: Urine samples collected sequentially in a prospective manner from 31 melanoma patients treated by curative surgery were analyzed for U-TAA level by the double determinant EIA as described in Example IX with the following modification. The samples were heated at 100°C in a boiling water bath for 2.5 min, cooled in an ice water

bath for 5 min. and mixed with equal volume (200 μ l) of 0.025M phosphate buffered saline supplement with 0.5% Tween-20. One hundred microliters of these mixtures were tested in the capture assay. Of the 31 patients, the U-TAA ELISA value of 10 patients remained negative (<0.68 OD₄₀₅) during the course of their monitoring for one year. Of these, only one (10%) has thus far had clinically detectable recurrence of the disease (Table 6). Recurrence in this patient occurred 24 weeks after entering the study. In contrast, the urine samples of 21 of 31 patients became U-TAA positive (>0.6 OD₄₀₅). Of these U-TAA positive patients, 12 (57%) have developed clinically detectable recurrence within 0 to 24 weeks). In view of the U-TAA positive results, this group of patients can be considered at high risk of recurrence. Analysis of sequential urine samples of patients with other malignancy can be used to monitor subclinical recurrence of the disease.

Table 6

Relationship between detection of U-TAA in urine of melanoma patients and recurrence of clinically detectable disease in a prospective study of one year.

U-TAA	# of Patients with recurrence	# of Patients without recurrence	Total
Negative	1	9	10
Positive	12	9*	21
		TOTAL	31

p <0.05 by students t-test

* patients with high risk of recurrence.

EXAMPLE XXX

Stimulation of U-TAA Expression by Interferons:

Modulation of the major histocompatibility antigens has been associated with tumorigenicity (Hayashi, H. et al.,
5 Cell 43:263-267, (1985) incorporated by reference herein). The evidence indicates that reduction or absence of class I antigen expression reduces immune recognition of tumor cells, allowing them to escape immune destruction. Treatment of tumor cells which have reduced or no
10 expression of class I HLA with interferon has resulted in reduced tumorigenicity (Tanaka, K.K. et al., Science (Wash. DC) 228:26-30 (1985); Eager, K.B. et al., Proc. Natl. Acad. Sci. USA 82:5525-5529 (1985) incorporated by reference herein).

15

It is known that treatment of human cultured tumor cells with interferon often increases the expression of histocompatibility antigens (class I) and modulates (increases or decreases) the expression of tumor antigens
20 (Imai, K. et al., J. Immunol. 127:505-509; Giacomini, P. et al., J. Immunol. 133:1649-1655; Perosa, F. et al., J. Immunol. 138:2202-2207 (1987) each of which is incorporated by reference herein). We treated three melanoma cell lines - UCLA-SO-M10, UCLA-SO-M24, and UCLA-SO-M101 - with gamma
25 and alpha-interferon to determine if this affected the level of U-TAA expression. The cells of each cell line were cultured in RPMI-10% FCS with and without supplementation with 500 units/ml of the interferons at 37° for 96 hrs. After treatment, the cells were harvested by
30 scrapping, washed twice with RPMI-10% FCS, and assessed for the amount of U-TAA. A competitive ELISA utilizing allogeneic antibody and purified U-TAA was used. The allogeneic antibody, before and after absorption with the varying number of melanoma cells which had been grown in
35 the presence and absence of interferon, was reacted with

the purified U-TAA. Purified U-TAA (0.06 $\mu\text{g/ml}$) was used as the standard blocker to generate the standard inhibition curve in the ELISA.

5 The expression of U-TAA by UCLA-SO-M10 and UCLA-SO-M101 cell lines was increased by growing the cells in the presence of either gamma interferon or alpha interferon. The expression of U-TAA by UCLA-SO-M24, however, was not affected by treatment with either interferon (Figure 10).
10 As a control, the expression of HLA-DR antigen was determined by reacting the cells with ^{125}I -labeled monoclonal antibody (anti-HLA-DR) in a suspension assay. Binding of radiolabeled antibody by cells of all three cultures grown in the presence of the interferons was significantly higher
15 (2 to 3 times) than those grown in its absence.

EXAMPLE XXXI

U-TAA is a Cell Surface Antigen: Randomly selected sera
20 from MCV patients who had demonstrated high anti-U-TAA antibody titers in ELISA were tested by immunofluorescence for antibodies capable of binding to the surface of cultured melanoma cells. Sera demonstrating strong membrane fluorescence were tested further by using the
25 indirect membrane immunofluorescence method to determine if U-TAA on the cell surface was responsible for part of this reactivity. Serum samples were serially diluted and incubated for 30 minutes at 37°C and 30 minutes at 4°C with equal volume (50 μl) of PBS alone (negative control) or PBS
30 with 1 μg of U-TAA. Cultured UCLA-SO-M 24 melanoma cells were harvested by treatment with versene (0.05 mM sodium EDTA, 0.014 M NaCl, 0.5 mM M KCl and 0.55 mM dextrose) and 0.25% trypsin. The harvested cells were washed 3 times with Hank's balanced salt solution (HBSS), containing 0.01%
35 human serum albumin and resuspended in HBSS-albumin at a

concentration of 5×10^4 cells/ml. Fifty microliters of the cell suspension were mixed with 50 μ l of melanoma serum at various dilutions and incubated at 37°C for one hour and at 4°C for one hour. The cells were washed 3 times with HBSS-
5 albumin and reacted with 50 μ l of fluorescein-conjugated goat anti-human immunoglobulin at 23°C for 20 minutes. After washing, the cells were resuspended in 25 μ l of 50% glycerol in 0.025 M PBS, placed on glass slides and examined for membrane immunofluorescence under a
10 fluorescent microscope. The presence of 3 or more fluorescent dots on the cell membrane was considered positive for antibody. Diffuse or cytoplasmic fluorescence was not regarded as positive. The percentage of positive cells was calculated following a 50 cell count.

15

Sera with high anti-U-TAA antibody titers when tested in indirect membrane immunofluorescence showed binding of antibody to UCLA-SO-M 24 cultured melanoma cells. One serum was selected for inhibition studies with U-TAA as
20 described above. This serum showed binding to 50-60% of melanoma cells even when diluted at 1:16. Preincubation of this serum with 1 μ g of purified U-TAA resulted in a 93% reduction in the number of cells exhibiting immunofluorescence at the 1:16 serum dilution (Figure 11).
25 The ability of U-TAA to inhibit the binding of high titer anti-U-TAA antibody in the indirect membrane immunofluorescence assay convincingly demonstrated the expression of U-TAA on the surface cell membrane of tumor cells.

30

EXAMPLE XXXII

In Vitro Production of Human Antibodies to U-TAA: The Ficol-hypaque centrifugation method was used to obtain
35 peripheral blood lymphocytes (PBL) from 30 ml of ~~heparinized~~ *heparinized*

blood of cancer patients. The blood donors, who were participants in the MCV trial, were selected on the basis of their high levels of anti-U-TAA antibody. By application of the erythrocyte rosetting technique (Bakacs et al., Cellular Immunology (1977) incorporated by reference herein), T-cells were removed from the isolated PBL, which were thereby enriched for B-cells (antibody producing cells). The enriched B-cells were transformed by infecting them with Epstein Barr virus (EBV). For this purpose, enriched B-cells were suspended at a density of 10×10^6 cells per ml in RPMI 1640-10% FCS containing 50% supernate of the EBV producing marmaset cell line, B95-8. After overnight incubation at 37°C, the cells were washed and resuspended in the RPMI-FCS medium. Supernates from these cultures were collected at weekly intervals and tested for the presence of anti-U-TAA activity in the direct ELISA, using purified U-TAA as the target antigen (Example I). Seventeen of 35 cultures produced antibodies to U-TAA, although many cultures stopped producing the antibodies within three months. Cells from two cultures that continued to produce the antibodies for more than six months (Figure 12) were cryopreserved at regular intervals.

To document that the immunoreactive components recognized by the antibodies in the supernates were expressed by tumor cells, a ten fold concentrated supernate of these cultures was used as the source of in vivo produced antibody to U-TAA. Melanoma cells (UCLA-SO-M14) were cultured for five days in RPMI supplemented with ^{14}C -L-leucine (50 uCi/ml). The cells were harvested, washed 3 times with complete RPMI-FCS medium, and extracted with 0.5% (V/V) NP-40 (~~Nonidet~~ ^{Nonidet} P-40) to obtain biosynthetically labeled antigen. The cell free extract was treated with immobilized wheat germ agglutinin (WGA). The bound material was eluted with chitotriase and was

chromatographed through a Sephacryl S-200 column (0.5 x 10cm), using 0.025M phosphate buffered saline as the eluent. Two hundred microliter fractions were collected. The elution profile was monitored by subjecting 10 μ l
5 aliquots to scintillation counting (Figure 13). Fractions under each peak of radioactivity were pooled and reacted with 100 μ l of 10 x concentrated human lymphoblastoid culture supernate (in vitro produced antibody source). The radioactivity bound to the antibody was sedimented with 200
10 μ l of 50% suspension of rabbit anti-human Ig immunobeads (Biorad Laboratories, Richmond, CA). As illustrated in Figure 13, about 65% radioactivity of pool of fractions 39 to 45 bound to the human antibodies that were present in the lymphoblastoid culture supernate.

15

Although we have not yet succeeded in developing clones from a single cell, we have succeeded in generating subclones from lymphoblastoid cell lines (LCL 1 and LCL 2) by culturing them in soft agar (1%) using thymocytes as the
20 feeder layers. Theoretically, colonies that develop in the soft agar are from single cells. However, this is not always true, because two or more cells often adhere to one another. Thus, monoclonality of such colonies can not be guaranteed. Analysis of two subclones (LCL 2.6 and LCL
25 2.11) of LCL2 culture revealed that they produce anti-U-TAA and anti-FA IgM antibodies.

The specificity of the immunoreactivity of culture supernates of subclones LCL 2.6 and 2.11 was confirmed by
30 blocking with purified FA and U-TAA and then reacting against the two target antigens in ELISA. The doubling dilution method was used to dilute serially the culture supernates. One aliquot (100 μ l) of each dilution was mixed with 0.025 M phosphate buffer supplemented with 0.15
35 M NaCl and 0.5% Tween-20 as an unblocked control; a second

aliquot was blocked with an equal volume (100 μ l) of FA (0.05 mg/ml); and a third aliquot was reacted with U-TAA (0.06 mg/ml). The mixtures were incubated at 37°C for one hour. Anti-U-TAA and anti-FA antibody levels in each of the mixtures to U-TAA and FA were determined by ELISA. Data presented in Figures 14 and 15 show that the reactivity of culture supernates of subclone LCL 2.6 to U-TAA was blocked by U-TAA and not by FA. Also, its reactivity to FA was blocked by FA and not by U-TAA. Similar results were observed when culture supernates of another subclone, LCL 2.11, were used as the source antibody. These results clearly show that, while the subclones LCL 2.6 and LCL 2.11 are not monoclonal at this point and time, they are producing anti-U-TAA (IgM) antibody. Subsequent subcloning of these subclones should produce clones that are monoclonal in nature.

EXAMPLE XXXIII

Detection of U-TAA that is Present in the Form of Immune Complexes in Sera of Cancer Patients: Because U-TAA is immunogenic in cancer patients, it elicits immune response. Thus, antibodies to U-TAA circulate in the blood of cancer patients, especially when the tumor burden is minimal. These antibodies may react with U-TAA on tumor cell surface. We have documented the presence of antibodies on tumor cell surfaces and determined that they react with tumor antigens specifically (Gupta, R.K. and Morton, D.L, Contemporary Topics in Immunobiology 15:1053 (1984) incorporated by reference herein). The tumor bound antibodies can be considered evidence for their immunologic interaction in vivo with corresponding antigens. The antigen-antibody (immune) complexes formed on the cell surface may be internalized by the cell or released into surroundings by the process of antigen modulation, capping,

and shedding (Leong S.P.L. et al., Cancer Res. 39:2125 (1979) incorporated by reference herein). Other possible mechanisms for release of tumor antigens into circulation include cell death, surface bleeding, sublethal autolysis, and secretion from cells (Price, M.R. and Robins, R.W., Immunological Aspects of Cancer, pp. 155 (1978) incorporated by reference herein). The antigens shed into circulation combine with humoral antibodies and result in the formation of circulating immune complexes. Thus, numerous efforts have been made by various investigators with some success to utilize immune complexes as a marker for immunodiagnosis and immunoprogno-
10 sis of human cancer. However, the antigenic nature of the complexes was unknown and correlations with the clinical course of the disease in cancer patients have not been unequivocal. This is mainly
15 because of the use of antigen-nonspecific immune complex detection assays.

With the availability of murine monoclonal antibody, AD1-40F4, that recognizes 90-100 kD subunit produced by human tumor cells, we have developed an antigen-specific immune complex detection assay. The assay, as illustrated in Figure 16, utilizes immobilization of the murine monoclonal antibody, AD1-40F4, to a solid matrix. The solid matrix could, for example, be polyvinyl or polystyrene microtiter plates or tubes or beads, Teflon discs, glass beads, or any other suitable material in any suitable physical form, e.g. glass fiber discs. The immobilization could, for example, be physical adsorption or covalent attachment by means of chemical reaction using
30 homo- or hetero-bifunctional or multifunctional coupling agents. The immobilized monoclonal antibody is reacted with serum sample or other body fluid suspected of being comprised of U-TAA (antigen)-anti-U-TAA (antibody) complexes, or purified immune complexes from serum or other
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body fluid. After washing the solid matrix, the presence of human immunoglobulin on its surface is realized by enzyme or biotin labeled antibody to human immunoglobulins, general or specific for each isotype, e.g. IgG, IgM, IgA, etc. Since the murine monoclonal antibody immobilized to the solid matrix does not react with any of the serum components, the presence of human immunoglobulins on the solid matrix is due to capture of the antigen that has already reacted with the patient's antibody in vivo. Upon exposure of the conjugate reacted solid matrix to appropriate substrate, a color development indicates the presence of the antigen (U-TAA) specific immune complexes in a test sample.

We have developed the above technique in polystyrene microtiter plates (Immulon I, Dynatech Laboratories, Chantilly, VA). Murine monoclonal antibody, AD1-40F4, was attached to the wells (100 μ l at 1:300 dilution per well). Test sera (100 μ l at 1:20 dilution) were added to the murine antibody sensitized wells and incubated at 37°C for 1.0 hr. After washing, the presence of human IgG (present in the form of immune complexes) in the test wells was determined by alkaline phosphatase conjugated goat anti-human IgG and p-nitrophenyl phosphate as the substrate. The results were expressed as optical density (OD) at 405 nm. Sera from 24 melanoma patients had a mean \pm SE OD₄₀₅ of 0.456 \pm 0.114. This value is significantly greater than the mean \pm SE of 0.1 \pm 0.025 for sera of 32 apparently healthy normal controls ($p < 0.001$). Using a value of greater than the mean plus 2 standard deviations (0.392 OD₄₀₅) of normal controls as an indication for the presence of U-TAA specific immune complexes, 37.5% (9/24) of the sera from melanoma patients was positive in contrast to only 6% (2/32) from normal controls ($P < 0.01$).

Analysis of sequentially collected serum samples during clinical course of 9 melanoma patients in a prospective study revealed that this U-TAA-specific immune complex detection assay could be used to identify patients with subclinical disease several months before their recurrence became clinically obvious. Figure 17 illustrates this fact using data from two representative patients. Positive levels of U-TAA-specific immune complexes were detected 4 to 24 weeks (mean = 14.1 weeks) before clinically detectable recurrence. Thus, this offers a useful means of identifying microscopic, subclinical disease by monitoring the presence of U-TAA-specific immune complexes in sera of surgically treated cancer patients.

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EXAMPLE XXXIV

Preparation of Human Anti-idiotypic Antibodies: Large quantities of an antigen are needed for therapeutic application. The amounts obtained from cultured tumor cell extracts or from the urine of cancer patients are only enough to use in in vitro serologic and immunochemical assays. To provide an adequate supply of antigen, two approaches are available: 1) clone the gene responsible for production of U-TAA through molecular biology techniques; 2) identify EBV-transformed lymphoblastoid cells from cancer patients or human-human hybridoma of these cells that secrete anti-idiotypic antibody (internal mirror image of anti-U-TAA antibody) of anti-U-TAA. These approaches can be used successfully by those skilled in the methodology. The second approach is preferable at this time because we already ^{have} had EBV-transformed lymphoblastoid cells and human-human hybridomas from lymphocytes of cancer patients.

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Many of these lymphoblastoid cell lines secrete

immunoglobulins but do not react with U-TAA. It is possible that some of these immunoglobulins may be anti-idiotypes of U-TAA. We have identified lymphoblastoid subclones that produce anti-U-TAA of IgM isotypes (Example
5 XXXII). Once large quantities of these antibodies are obtained in purified form, they can be conjugated to enzymes or other suitable ~~radionuclides~~ ^{radio-nuclides} and used for their ability to react with supernates of lymphoblastoid clones that do not react with U-TAA to identify clones with
10 possible anti-idiotypic reactivity. Production of anti-idiotypic antibodies to U-TAA by the positive clones can be confirmed by the blocking of anti-U-TAA with U-TAA before reacting with the suspected anti-idiotypic antibody producing clone supernates. To rule out the detection of
15 anti-idiotypes other than internal mirror image of U-TAA, the labeled antibody will contain purified human normal serum immunoglobulin (2% V/V). The anti-idiotypes identified in this manner have a vast application for active specific immunotherapy and for chemical analysis of
20 antigenic epitopes of U-TAA, e.g. amino acid sequence, nucleotide sequence, etc.